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ON THE MECHANISM OF CONTRACTION AND DESENSITIZATION INDUCED BY SUBSTANCE P IN THE INTESTINAL MUSCLE OF THE GUINEA-PIG

BY P. HOLZER AND ULRIKE PETSCHE

From the Department of Experimental and Clinical Pharmacology, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria

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SUMMARY

1. The contractile effect of substance P on the longitudinal muscle of the isolated guinea-pig small intestine and the desensitization of the muscle which occurs on prolonged exposure to the peptide have been investigated. All experiments were performed in the presence of atropine.

2. The response to a substance P concentration which produced a nearly maximal effect was not sustained but faded rapidly. It was found that not elimination of substance P from the bath, but desensitization of the muscle to substance P was the main cause for the fading of contraction.

3. Desensitization of the muscle to substance P only developed if the muscle was exposed to the peptide for a certain time. The degree of and the time needed for recovery from desensitization were directly related to concentration of substance P and contact time.

4. Tetraethylammonium (3 mM), which reduces the membrane conductance for K^+, enhanced the potency of substance P in contracting the muscle and reduced the fading of contraction. Noradrenaline (295 nM), which increases the K^+ conductance, produced opposite effects.

5. Lowering the extracellular Ca^{2+} concentration to one-tenth decreased the potency of substance P in contracting the muscle, accelerated the fading of contraction, and reduced the ability of the muscle to respond to a second addition of substance P after the response to the first addition had faded away.

6. Concentrations of substance P (22 nM) and tetraethylammonium (30 mM), which produced nearly maximal contractions, slightly enhanced the efflux of ^{86}Rb from pre-loaded muscle strips. Both substances, however, caused a sustained reduction of ^{86}Rb efflux from strips depolarized by high $[K^+]$, the effect of substance P being smaller than that of tetraethylammonium. The effect of substance P and tetraethylammonium on ^{86}Rb efflux appeared independent of the extracellular $[Ca^{2+}]$.

7. On exposure of the muscle to substance P (22 nM) for 8 min the intracellular uptake of ^{45}Ca was first decreased and then increased while the ^{45}Ca influx was instantly enhanced by tetraethylammonium (30 mM) or K^+ (108 mM). The delayed increase in ^{45}Ca influx caused by substance P was also observed in muscle strips depolarized with high $[K^+]$.

8. Taken together, the results suggest that there are two mechanisms of action by which substance P causes contraction of intestinal smooth muscle: reduction of a K^+ conductance and a mechanism dependent on the extracellular [Ca^{2+}]. Circumstantial evidence indicates that desensitization of the muscle to substance P arises from a block in excitation–contraction coupling and the hypothesis has been put forward that fading of the contractile response and desensitization reflect depletion of a Ca^{2+} store which is operated by the substance P receptor.

INTRODUCTION

Substance P has been localized to both neurones and endocrine cells of the mammalian intestine (Nilsson, Larsson, Håkanson, Brodin, Pernow & Sundler, 1975; Pearse & Polak, 1975; Costa, Cuello, Furness & Franco, 1980; Schultzberg, Hökfelt, Nilsson, Terenius, Rehfeld, Brown, Elde, Goldstein & Said, 1980). The peptide appears to be involved in the communication from neurone to neurone and neurone to muscle, since there is evidence that on release from nerve endings substance P excites both neurones (Katayama, North & Williams, 1979; Holzer & Lembeck, 1980; Morita, North & Katayama, 1980; Leander, Håkanson, Rosell, Folkers, Sundler & Tornqvist, 1981) and intestinal smooth muscle (Franco, Costa & Furness, 1979; Holzer, Lembeck & Donnerer, 1980). The contractile effect of substance P on the longitudinal muscle of the guinea-pig small intestine is mainly due to a direct action on the muscle cells (among others Bury & Mashford, 1977; Rosell, Björkroth, Chang, Yamaguchi, Wan, Rackur, Fisher & Folkers, 1977; Franco *et al.* 1979); however, release of acetylcholine seems to contribute to maintaining the contractile response to substance P (Holzer & Lembeck, 1980; Holzer, Emson, Iversen & Sharman, 1981). Circumstantial evidence indicates that the mechanism underlying the direct contractile effect of substance P is inactivation of the resting K^+ conductance (Fujisawa & Ito, 1982); a similar mechanism, reduction of a potential-sensitive K^+ conductance, has been proposed to account for the facilitatory effect of substance P on the phasic contractions of the rabbit small intestine (Holzer, 1982).

In a communication to this journal, Gaddum (1953) was the first to observe that high concentrations of a crude substance P preparation rendered the guinea-pig ileum insensitive to further additions of the extract. The desensitizing effect of substance P was further studied by Lembeck & Fischer (1967), and with the use of synthetic substance P it was shown that desensitization to substance P is specific and can be used to study the release of endogenous substance P from the intestine (Franco *et al.* 1979). The contractile response to substance P, particularly in high concentrations, is not sustained but rapidly fades, and fading of the contraction has been considered to reflect ongoing desensitization (Holzer & Lembeck, 1980; Jordan, 1980; Holzer *et al.* 1981; Huidobro-Toro, Chelala, Bahouth, Nodar & Musacchio, 1982). It has also been suggested that desensitization could be a means by which the transmission of impulses from substance P neurones to muscle cells is terminated (Holzer *et al.* 1981). An examination of desensitization to substance P on the basis of a desensitization model proposed by Rang & Ritter (1970) yielded ambiguous results (Jordan, 1980); thus there is still no information as to processes that cause desensitization.

The present study was aimed at elucidating (i) the mechanisms by which substance

P causes contraction of the longitudinal muscle of the isolated guinea-pig small intestine, (ii) the mechanisms by which intestinal smooth muscle is rendered insensitive to substance P, and (iii) the relationship which might exist between the mechanisms of action and the mechanisms of desensitization. All experiments were performed in the presence of atropine so as to avoid effects of acetylcholine which might be released by substance P.

METHODS

Recording of contractions

Tissue preparation. Adult guinea-pigs, of 300–500 g body weight and either sex, were killed by decapitation. The small intestine was excised rapidly, about 5 cm of the terminal ileum being discarded, and kept in Tyrode solution, gassed with a mixture of 95% O_2 and 5% CO_2, at room temperature for no longer than 4 h.

Recording. Segments of the ileum, 1·5–2·0 cm in length, were suspended in a 7 ml glass organ bath (not silanized) which contained aerated Tyrode solution and was maintained at 37 °C. The preparations were kept under a resting load of 0·5 g, and longitudinal contractions of the ileum were recorded isotonically by means of a lever displacement measuring system (Hugo Sachs Elektronik) and displayed on a Rikadenki pen recorder.

Solutions. The standard Tyrode solution was of the following composition (mM): NaCl, 136·9; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1·0; $NaHCO_3$, 11·9; NaH_2PO_4, 0·4; glucose, 5·6; and atropine 0·0006; pH 7·4. In some experiments, the Ca^{2+} concentration was reduced to one-tenth or increased by a factor of 5, the medium being kept isosmotic by changing the Na^+ concentration. In other experiments, 3 mM-tetraethylammonium or 295 nM-noradrenaline were added to the Tyrode solution. In each of these experiments the ileum was allowed to equilibrate in the new medium for 30 min during which the bath was washed repeatedly.

Protocol. Experiments were begun after an equilibration period of 30 min during which the bath medium was changed repeatedly. Then the tissue was challenged three times with maximally effective concentrations of histamine (9 μM) to standardize the preparation. After a further 10 min the experiments with substance P were started by establishing the concentration of substance P that consistently caused a contraction being about 50% of that caused by 9 μM-histamine (EC_{50}). This often took another 30 min during which the sensitivity of the tissue to substance P gradually increased but then remained constant for at least 2 h. Unless otherwise stated, the spasmogens used in this study were left in contact with the tissue until a peak response was established (usually within 20 s) and then removed by three washings. Only one of the experiments described below was carried out with each preparation. Contractile responses to substance P were expressed as a percentage of the maximal response to histamine in standard medium; histamine was chosen, since the maximal contractile effects of substance P and histamine are equal (see Bury & Mashford, 1976; Rosell *et al.* 1977).

Conventional concentration–response curves were generated by exposure of the ileum to increasing concentrations of substance P at 3 min intervals, each addition of substance P being followed by a wash after the response had reached a peak. After recording a concentration–response curve in this way the bath medium was changed (see above), the ileum allowed to equilibrate, and a second concentration–response curve recorded. *Cumulative concentration–response curves* were established by adding increasing doses of substance P to the bath without washings in between. The doses were added at 0·5, 2·0 or 4·0 min intervals.

In order to study the *desensitization* of the ileum to substance P and the *recovery from desensitization* the EC_{50} of substance P was used as *test concentration*, and a multiple of the EC_{50} as *desensitizing concentration*. The ileum was exposed to the desensitizing concentration for 30, 100 or 300 s; thereafter the ileum was exposed to the EC_{50} at 3 min intervals until the pre-desensitization response was re-established, the first addition being made 2·5 min after washout of the desensitizing concentration. The same protocol was adhered to in order to study the *specificity of desensitization*; in this instance, substance P, histamine, tetraethylammonium, and KCl were used as test substances, and substance P and tetraethylammonium as desensitizing substances. The after-desensitization responses to the test substances were expressed as a percentage of the pre-desensitization response (100%) to the respective substances.

In order to study the *fading of the contractile response* to substance P segments of ileum were exposed to ten times the EC_{50} of substance P, and 5 and 10 min after the first addition of substance P the same dose was added again without previous washing. These experiments were repeated in altered bath media (see above). The contractions were expressed as a percentage of the response to 9 μM-histamine in the standard Tyrode solution.

Determination of substance P by radioimmunoassay

In order to relate the time course of the contractile response to the actual concentration of substance P in the bath the following experiments were performed. Substance P (74 nM) was added to the bath at time 0; at 15 s, 1·5 and 5·0 min samples of 0·1 ml bath fluid were withdrawn, acidified with acetic acid to pH 3·5 and freeze-dried. Their substance P content was measured by radioimmunoassay according to Mroz & Leeman (1979). The same experiment was conducted in Tyrode solution containing either 1 g/l or no gelatin and in the presence and absence of ileal tissue in the organ bath. Separate experiments were concerned with the effect of silanizing the glass bath on the elimination of 74 nM-substance P from the bath fluid. For this purpose the glass bath was dipped in Surfasil siliconizing fluid (Pierce; 10% (v/v) in carbon tetrachloride) for 10 min and air-dried.

Ion flux measurements

Tissue preparation. Strips of longitudinal muscle with adhering myenteric plexus from the jejunum and ileum of the guinea-pig were prepared as described by Bolton (1972).

Solutions. The standard Tyrode solution was of the same composition as described above, but in addition also contained 0·31 μM-tetrodotoxin to block propagated neural activity in the myenteric plexus. In the experiments concerned with ^{86}Rb efflux 1 mM-RbCl was added to the media in order to reduce non-specific binding of ^{86}Rb. If the K^+ concentration was increased by a factor of 40, the medium was kept isosmotic by an appropriate change of the Na^+ concentration. Throughout the experiments the media were maintained at 37 °C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2.

Efflux of ^{86}Rb. The strips were equilibrated for 20 min and then incubated for 2–4 h in standard Tyrode solution containing 0·37 MBq ^{86}Rb/ml (specific activity: 10 MBq/μmol; Amersham). After loading, the strips were transferred to the efflux chambers (200–300 mg tissue per chamber) which contained 2 ml non-radioactive medium. The bottoms of the chambers were of nylon gauze through which the medium could be collected by suction, the system being similar to that used by Sharman, Holzer & Holzbauer (1982).

The medium was collected every 2 min and replaced by fresh medium within 10 s. The ^{86}Rb content of the efflux samples and of the tissues at the end of the experiment was determined by gamma counting. The efflux rate constant was calculated according to the formula $\Delta A/\Delta t \,.\, A_t$, where ΔA represents the counts lost in the time interval Δt and A_t is the number of counts contained in the tissue at the mid point of the interval Δt (Burgen & Spero, 1968).

Influx of ^{45}Ca. Measurements of ^{45}Ca influx and cellular ^{45}Ca content were carried out according to the methods described by Aaronson & van Breemen (1981). Following 1 h equilibration in standard Tyrode solution, strips (25–40 mg) were equilibrated for a further 45 min period in the respective experimental media. For measurement of *unidirectional ^{45}Ca influx*, strips were placed in experimental media containing 37 kBq ^{45}Ca/ml (specific activity: 24 MBq/μmol; Amersham) for 2 min. During this short time period ^{45}Ca uptake is almost entirely a function of ^{45}Ca influx, ^{45}Ca efflux being negligible (Aaronson & van Breemen, 1981). After the 2 min 'pulse labelling' period the strips were immediately placed into an ice-cold, vigorously bubbled solution identical to the standard Tyrode solution except that Ca was omitted and 2 mM-ethyleneglycol-bis-(β-aminoethylether) *N*, *N'*-tetraacetic acid (EGTA) was added. The purpose of this wash was to remove all extracellular bound ^{45}Ca while leaving intracellular ^{45}Ca stores intact (van Breemen, Aaronson & Loutzenhiser, 1980).

After a 45 min wash the strips were blotted briefly and weighed, and left overnight in scintillation vials containing 3 ml 5 mM-ethylenediaminetetraacetic acid (EDTA) to disperse ^{45}Ca into the vial solution. Subsequently, 7 ml scintillator (Unisolve I, Koch-Light Laboratories) was added to each vial and the ^{45}Ca content of each tissue measured by beta counting.

The pulse labelling method was used to study the ^{45}Ca influx at various times during an 8 min exposure to substance P, tetraethylammonium or elevated K^+ concentration. To investigate

whether exposure to these substances for 8 min has an effect on the *intracellular Ca content*, strips were equilibrated in standard Tyrode solution for 1 h and then placed into experimental media containing 37 kBq ^{45}Ca/ml (loading media) for another hour. Tissues were then removed to experimental media containing either substance P, tetraethylammonium or elevated K^+ concentration, each containing the same specific activity of ^{45}Ca as the loading medium, so that any changes in tissue ^{45}Ca were due to a net uptake or loss of ^{45}Ca by the tissue. After 8 min tissues were removed from the labelled media, washed in EGTA solution and processed to quantitate cellular ^{45}Ca as described above.

Substances

Substance P was obtained from Peninsula, tetrodotoxin from Sigma, and tetraethylammonium chloride from Merck-Schuchardt. All other chemicals were of analytical purity and purchased from commercial sources. Substance P was dissolved in 0·1 M-acetic acid (1 mg/ml) and dilutions made with Tyrode solution containing 1 g/l gelatin to reduce adsorption to surfaces. All other substances were dissolved in water and dilutions made with Tyrode solution.

Statistics

All values are expressed as the mean and S.E. of the mean. Student's t test or one way analysis of variance together with the Duncan test were used for statistical comparisons, P values $< 0·05$ being regarded as significant.

RESULTS

Bell shape of cumulative concentration–response curves for substance P

The concentration–response curve (contraction *versus* log concentration) for the contractile effect of substance P was sigmoid, when it was recorded by the conventional procedure (Fig. 1*A*) or when the doses were cumulatively added at 0·5 min intervals (Fig. 1*A* and *B*). However, when the time intervals at which the doses were cumulatively added were gradually increased to 2 and 4 min, the shape of the concentration–response curve changed from sigmoid to bell-like and the maximal effect of substance P became smaller (Fig. 1*A*). A tracing of a cumulative concentration–response curve with doses added at 4 min intervals (Fig. 1*B*) illustrates that with increasing doses of substance P not only the velocity of the contraction but also that of the fading of the contraction increased.

Effects of low Ca^{2+} concentration, tetraethylammonium and noradrenaline on the contractile effect of substance P

The contractile effect of substance P was investigated in standard Tyrode solution and in a medium containing either 3 mM-tetraethylammonium or 295 nM-noradrenaline or whose Ca^{2+} concentration had been reduced to one tenth. Exposure of the ileum to 3 mM-tetraethylammonium resulted in contraction and increased spontaneous activity; while the contraction completely waned during the 30 min equilibration period the spontaneous activity remained increased. Low medium [Ca^{2+}] reduced the contractile response to 9 μM-histamine to $79 \pm 2\%$ ($n = 11$, $P < 0·001$), 3 mM-tetraethylammonium reduced it to $97 \pm 1\%$ ($n = 6$, $P < 0·05$), and 295 nM-noradrenaline reduced it to $95 \pm 1\%$ ($n = 6$, $P < 0·01$) of the response in the standard Tyrode solution (paired t test). Fig. 2 shows that in low [Ca^{2+}] or 295 nM-noradrenaline the concentration–response curve for substance P was shifted to the right whereas in 3 mM-tetraethylammonium it was shifted to the left. All points of the concentration–response curves were significantly different from the respective

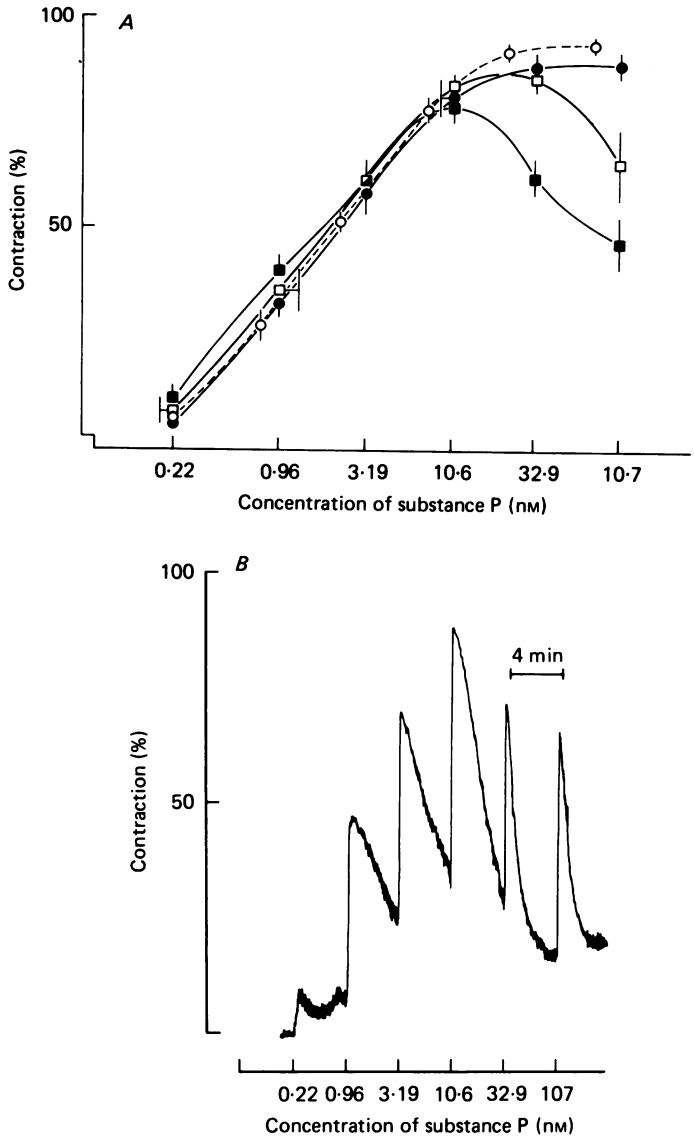

Fig. 1 *A*, concentration–response curves for the contractile effect of substance P on the isolated guinea-pig ileum, recorded with different protocols. Doses were either added at 3 min intervals and washed out as soon as a peak response was established (○) or cumulatively added at intervals of 0·5 min (●), 2 min (□) or 4 min (■). Means ± S.E. of means; $n = 6$. *B*, tracing showing the contractile response to increasing doses of substance P added cumulatively at 4 min intervals. Contractions are expressed as a percentage of the response to 9 μM-histamine.

points obtained in the standard medium ($P < 0·05$, paired t test). In low medium [Ca^{2+}] the concentration–response curve was flatter and the maximal effect of substance P clearly smaller than in the standard medium. Fig. 2 also shows that the tissue sensitivity to substance P varied from one series of experiments to the other, the EC_{50} values ranging from 1 to 5 nM.

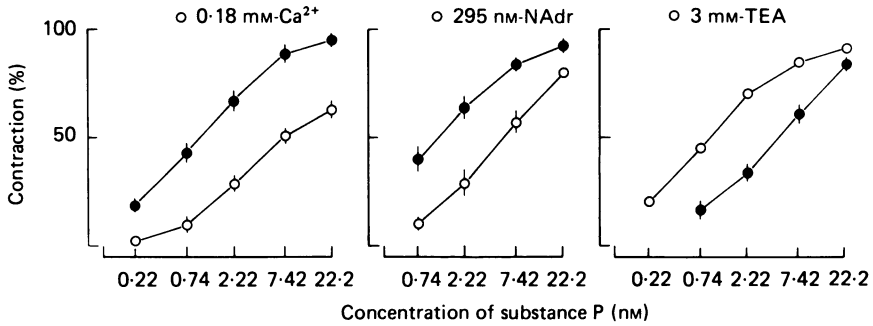

Fig. 2. Relation between concentration of substance P and longitudinal contraction of the isolated guinea-pig ileum in standard medium (1·8 mM-Ca^{2+}; ●) and in media containing 0·18 mM-Ca^{2+}, 295 nM-noradrenaline (NAdr) or 3 mM-tetraethylammonium (TEA). Contractions are expressed as a percentage of the response to 9 μM-histamine in the standard medium at the beginning of the experiment. Means ± S.E. of means; $n = 6$.

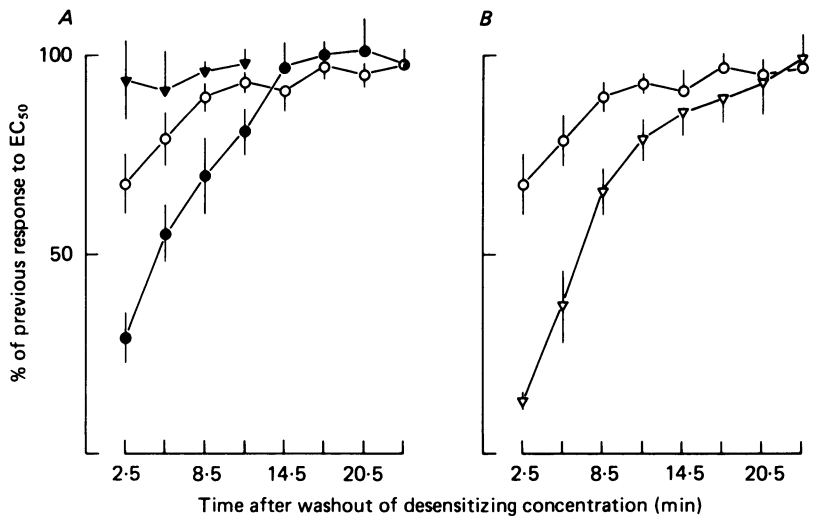

Fig. 3. *A*, relation between time of exposure to a desensitizing concentration of substance P ($10 \times EC_{50}$) and recovery from desensitization, determined by the contractile responses of the isolated guinea-pig ileum to the EC_{50} of substance P. Tissues were exposed to the desensitizing concentration of substance P for 30 s (▼), 100 s (○), and 300 s (●) and tested for sensitivity to substance P at the times indicated. *B*, relation between magnitude of the desensitizing concentration of substance P and recovery from desensitization. Tissues were exposed to $10 \times EC_{50}$ (○) and $100 \times EC_{50}$ (▽) of substance P as desensitizing concentrations and tested at the times indicated. Means ± S.E. of means; $n = 6$.

Desensitization of the ileum to substance P

Dependence on time of exposure to and concentration of substance P. The degree of desensitization, as judged by the reduction of the contractile response to the test concentration of substance P (EC_{50}), and the time needed for recovery from desensitization were directly related to the time of exposure to the desensitizing concentration ($10 \times EC_{50}$; Fig. 3*A*). Exposure to ten times the EC_{50} for only 30 s was too little a time span for rendering the ileum less sensitive to substance P. This fact

was confirmed by two other experiments (data not shown). Thus, if the test dose of substance P was given already 30 s after washout of the desensitizing concentration, the test response was reduced by only $9 \pm 5\%$ ($n = 7$, not significant). Likewise it was possible to get a stable response to ten times the EC_{50} when the ileum was challenged with this concentration at 1 min intervals, the exposure time being 30 s ($85 \pm 2\%$ of maximal contraction on first exposure, $86 \pm 4\%$ on second exposure, $n = 6$). Fig. 3*B* illustrates that the degree of desensitization was also dependent on the concentration of substance P that was used for desensitization.

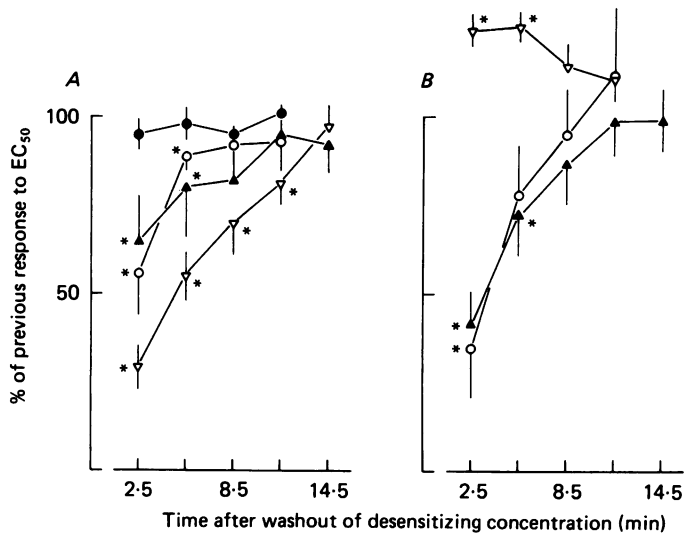

Fig. 4. Effect of desensitization of the isolated guinea-pig ileum to substance P (*A*) or tetraethylammonium (*B*) on the contractile responses to substance P (▽), tetraethylammonium (▲), KCl (○), and histamine (●). Tissues were exposed to $10 \times EC_{50}$ of substance P (*A*) or tetraethylammonium (*B*) as desensitizing concentrations and challenged with the EC_{50} of the respective substances at the times indicated. Means ± S.E. of means; $n = 6$; $^*P < 0{\cdot}05$ (compared with pre-desensitization response; paired *t* test).

In other experiments (data not shown) it was investigated whether the degree of desensitization varies if the test concentration were the EC_{30} or EC_{70} and the desensitizing concentration ten times the respective test concentration, the time of exposure to the desensitizing concentration being 5 min. It was found that there was no significant difference in the degree of desensitization and the time needed for recovery from desensitization: the response to the EC_{30} was reduced to $16 \pm 8\%$ ($n = 7$) and that to the EC_{70} to $13 \pm 5\%$ ($n = 6$) of the pre-desensitization response. Thus the extent of desensitization did not vary with various desensitizing concentrations if the ratio of desensitizing to test concentration was kept constant.

Specificity. Fig. 4*A* shows that the test responses to substance P, but also those to KCl and tetraethylammonium were reduced if the ileum had been desensitized to substance P. The test responses to KCl and tetraethylammonium were, however, less diminished than those to substance P. The test response to histamine remained unaltered in preparations rendered insensitive to substance P. Conversely, if tetraethylammonium was used as desensitizing substance (Fig. 4*B*) the test responses to

tetraethylammonium and KCl were decreased while the test responses to substance P were significantly enhanced. The EC_{50} for KCl was in the range of 10–25 mM, that of tetraethylammonium 1·5–4 mM, and that of histamine 70–200 nM, compared with 1–5 nM for substance P. While the contractile response to 10 times the EC_{50} of substance P reached a peak and then rapidly faded within 5 min (see Fig. 6) the responses to histamine and tetraethylammonium were sustained, the fading being less than 15% within 5 min.

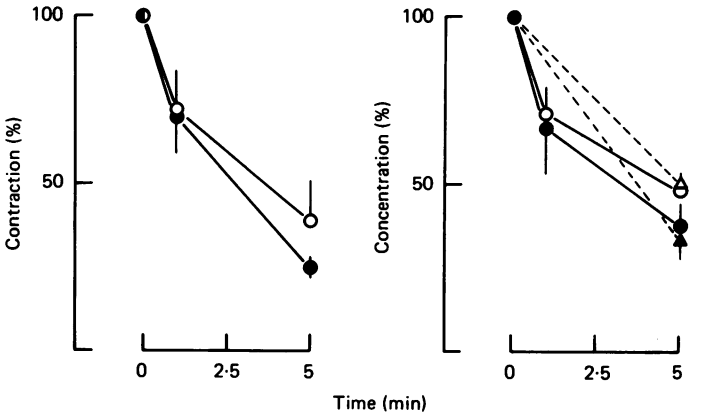

Fig. 5. Time course of the longitudinal contraction of the isolated guinea-pig ileum in response to 74 nM-substance P (left) and of the substance P concentration in the organ bath (right) under various conditions. Contractile response to and concentration of substance P were simultaneously measured in the presence (○) and absence (●) of gelatin (1 g/l), and concentration was also measured with no tissue present in the bath (△ with gelatin, ▲ without gelatin). Substance P was determined by radioimmunoassay. Values are expressed as a percentage of the initial peak contraction and concentration, respectively. Means ± S.E. of means; $n = 4$.

Fading of the contractile response to substance P

Relation to elimination of substance P. Fig. 5 compares the time course of the contractile response to 74 nM-substance P with that of the actual concentration of substance P in the bath as measured by radioimmunoassay. The contractile response underwent rapid fading within 5 min and the extent of fading was not significantly different in the presence and absence of 1 g/l gelatin although gelatin tended to diminish the fading of the contraction. The time course of the substance P concentration in the bath indicated that substance P rapidly disappeared from the bath. Fifteen seconds after addition of substance P, i.e. at a time when the contraction had reached a peak, the concentration was close to the value expected (76 ± 10 nM in medium without gelatin, 79 ± 7 nM in medium with 1 g/l gelatin, $n = 4$). Within the following 5 min the concentration fell by more than 50% and this was also observed when no tissue was present in the bath. The presence of 1 g/l gelatin tended to reduce the inactivation of substance P, but this tendency did not reach statistical significance. Silanizing the bath did not reduce the inactivation of substance P either: In the silanized bath the substance P concentration fell by 57 ± 4% ($n = 4$) within 5 min compared to 60 ± 3% ($n = 4$) in the untreated bath.

Effects of low Ca^{2+} concentration, noradrenaline and tetraethylammonium. Low [Ca^{2+}] or 295 nM-noradrenaline caused the contraction elicited by substance P to fade more rapidly and to a greater extent than in the standard medium. As shown in Fig. 6, the ileum completely relaxed within 5 min of exposure to ten times the EC_{50} of substance P. In contrast, tetraethylammonium (3 mM) caused the fading of the contraction to slow down and reduced the extent of the fading (Fig. 6).

When, 5 min after the first addition of substance P, i.e. at a time when the response had faded away, the same dose was added again without previous washing the ileum responded with a contraction that was much smaller than on first addition. The relative increase in contraction following a second and third addition of substance

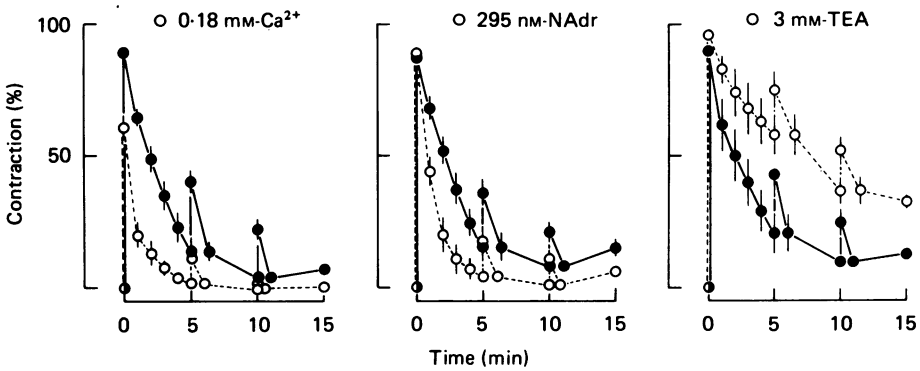

Fig. 6. Time course of longitudinal contractions of the isolated guinea-pig ileum induced by substance P in standard medium (1·8 mM-Ca^{2+}; ●) and in media (○) containing 0·18 mM-Ca^{2+}, 295 nM-noradrenaline (NAdr), or 3 mM-tetraethylammonium (TEA). Substance P ($10 \times EC_{50}$) was applied at times 0, 5 and 10 min. Contractions are expressed as a percentage of the response to 9 μM-histamine in standard medium. Means ± S.E. of means; $n = 6$–10.

P, 5 and 10 min after the first addition, was unchanged in 3 mM-tetraethylammonium, tended to be reduced, although without statistical significance, in 295 nM-noradrenaline, and was substantially suppressed ($P < 0{\cdot}01$) in low [Ca^{2+}] (paired t test; Fig. 6).

Effects of substance P and tetraethylammonium on ^{86}Rb efflux

Immediately after removing the strips of longitudinal muscle from the loading to the efflux medium the preparations rapidly lost some isotope, but within 15 min the rate constant of ^{86}Rb efflux settled down to a steady value around 0·014 min^{-1} (see Fig. 7) and then remained constant for up to 2 h. Drugs were therefore added 30 min after changing from the loading to the efflux medium. In standard Tyrode solution, substance P and tetraethylammonium caused a slight and sustained increase in ^{86}Rb efflux (Fig. 7). The increase was 1·14-fold for 22 nM-substance P ($n = 6$) and 1·20 fold for 30 mM-tetraethylammonium ($n = 6$) but 1·55-fold for 2·7 μM-histamine ($n = 5$, data not shown); all these effects were statistically significant ($P < 0{\cdot}01$, paired t test). The concentrations employed were equivalent to about ten times the EC_{50} for the contractile effect of the substances. Higher concentrations of substance P produced a somewhat higher increase in ^{86}Rb efflux: the increase was 1·20-fold for 74 nM-

substance P ($n = 9$) and 1·25-fold for 220 nM-substance P ($n = 5$, data not shown). These effects, however, were not statistically different from the effect of 22 nM-substance P (one way analysis of variance and Duncan test).

An increase in the K^+ concentration of the efflux medium from 2·7 to 108 mM caused the rate constant of ^{86}Rb efflux to increase by a factor of 3 (Fig. 7*B*). Under these conditions, both substance P (22 nM) and tetraethylammonium (30 mM) decreased the efflux of ^{86}Rb significantly ($P < 0·001$, paired t test), but tetraethylammonium was more effective in so doing than substance P (Fig. 7). While the rate constant

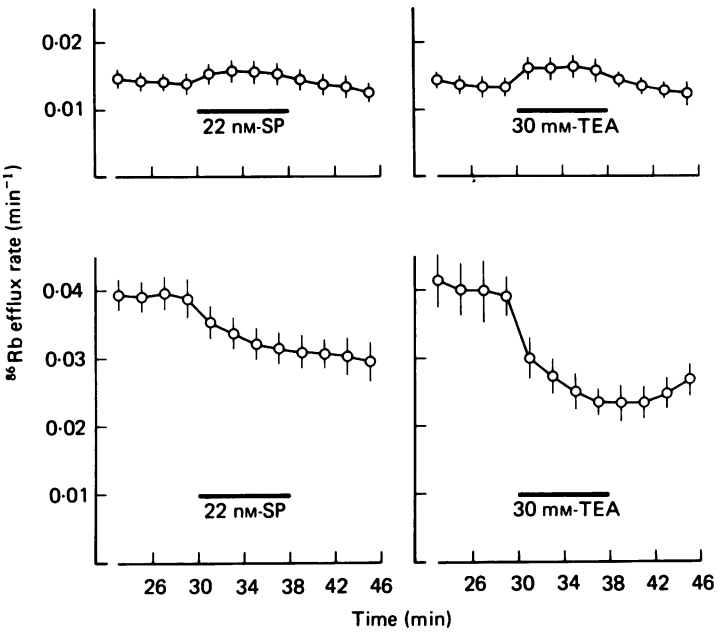

Fig. 7. Effect of substance P (SP) and tetraethylammonium (TEA) on efflux of ^{86}Rb from longitudinal muscle strips of the guinea-pig small intestine into standard medium (2·7 mM-K^+; top panels) or K^+-rich medium (108 mM-K^+; bottom panels). The substances were applied for the periods indicated by the horizontal bars. Time is minutes after transfer from loading to efflux medium. Means ± S.E. of means; $n = 6$.

tended to recover after exposure to tetraethylammonium for 8 min no sign of recovery was seen after exposure to substance P for 8 min.

The efflux of ^{86}Rb from longitudinal muscle strips, depolarized with high [K^+], was left unchanged by decreasing the Ca^{2+} concentration of the efflux medium to one tenth (0·18 mM) or by increasing it to 9·0 mM. The decreases in the ^{86}Rb efflux rate brought about by substance P (22 nM) or tetraethylammonium (30 mM) remained also unaffected (Table 1).

Effects of substance P and tetraethylammonium on ^{45}Ca uptake

Unidirectional ^{45}Ca influx. Longitudinal muscle strips were exposed to 22 nM-substance P or 30 mM-tetraethylammonium for 8 min and the ^{45}Ca influx rates determined during the first and last two minutes of this period by the pulse labelling method. Fig. 8*A* compares the effects of substance P and tetraethylammonium on

TABLE 1. Effect of substance P (22 nM) and tetraethylammonium (30 mM) on the rate constant of ^{86}Rb efflux (min^{-1}) from longitudinal muscle strips depolarized with 108 mM-K^+ in efflux media of varying [Ca^{2+}]. 'Before': efflux rate in the 2 min period before exposure to the substances; 'after': efflux rate in the last 2 min period of an 8 min exposure to the substances. Means ± S.E. of means, $n = 5$

	0·18 mM-Ca^{2+}	1·8 mM-Ca^{2+}	9·0 mM-Ca^{2+}
Before Substance P	0·0495 ± 0·0024	0·0463 ± 0·0036	0·0515 ± 0·0011
After	0·0419 ± 0·0029	0·0369 ± 0·0037	0·0416 ± 0·0016
Before Tetraethylammonium	0·0455 ± 0·0027	0·0447 ± 0·0046	0·0479 ± 0·0020
After	0·0287 ± 0·0005	0·0272 ± 0·0027	0·0307 ± 0·0011

Neither the efflux rates before addition of the substances nor the decreases in the efflux rate produced by the substances were significantly different in the different media (one way analysis of variance and Duncan test).

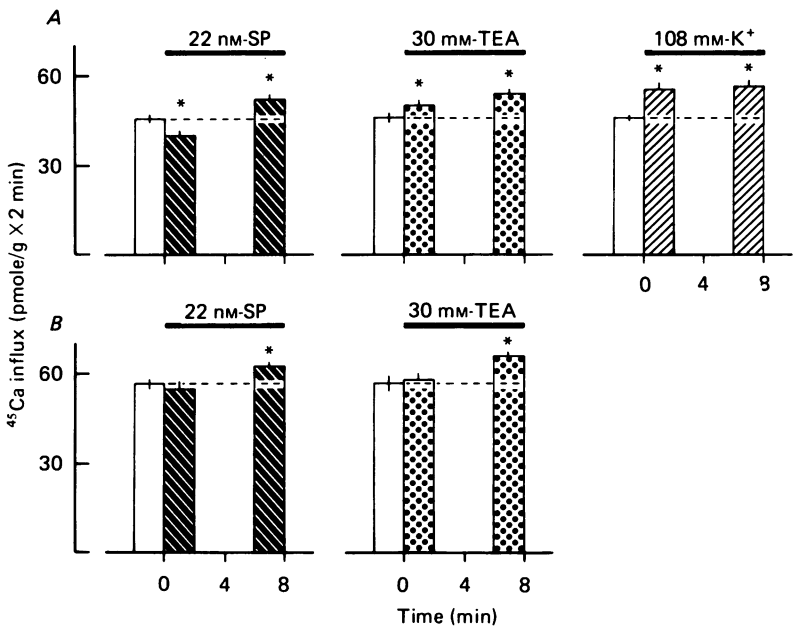

Fig. 8. Effect of substance P (SP), tetraethylammonium (TEA), and potassium (K^+) on ^{45}Ca influx into longitudinal muscle strips of the guinea-pig small intestine in standard medium (2·7 mM-K^+; *A*) or K^+-rich medium (108 mM-K^+; *B*). ^{45}Ca influx was measured during a 2 min ^{45}Ca labelling period at the beginning and end of an 8 min exposure to the substances. Means ± S.E. of means; $n = 8$–16; $*P < 0·05$ (compared with control ^{45}Ca influx; one way analysis of variance and Duncan test).

^{45}Ca influx with that of increasing the K^+ concentration from 2·7 to 108 mM. Following exposure to high [K^+] the ^{45}Ca influx rate immediately increased by a factor of 1·21 and remained elevated throughout the presence of high [K^+]. Tetraethylammonium had a similar effect on ^{45}Ca influx as high [K^+] but the effect developed gradually, the influx being increased 1·09-fold during the first and 1·17-fold during the last two minutes of exposure ($P < 0·01$, one way analysis of variance and Duncan

test). The effect of substance P on ^{45}Ca influx was quite different from that of high $[K^+]$ or tetraethylammonium, as the ^{45}Ca influx was decreased by a factor of 0·88 during the first but increased by a factor of 1·13 during the last two minutes of exposure.

Bathing the strips in 108 mM-K^+ solution for 45 min resulted in a 1·24 times larger ^{45}Ca influx than in standard Tyrode solution (Fig. 8*B*). Substance P or tetraethylammonium had no effect during the first but produced a 1·10- and 1·15-fold, respectively, increase in ^{45}Ca influx during the last two minutes of an 8 min exposure to these substances.

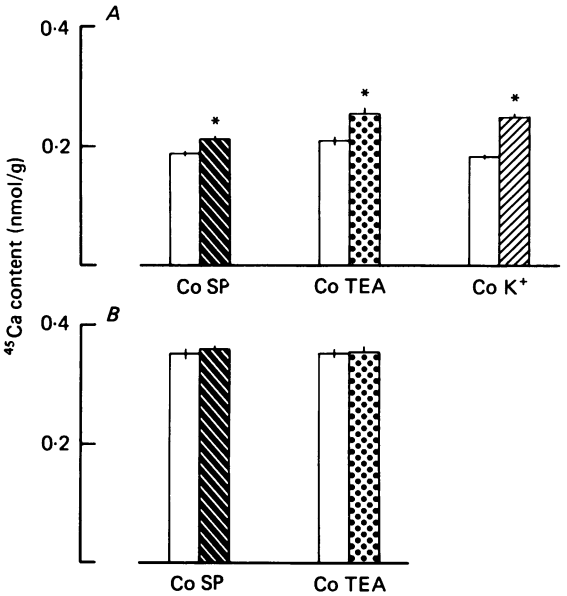

Fig. 9. Effect of substance P (SP; 22 nM), tetraethylammonium (TEA; 30 mM), and potassium (K^+; 108 mM) on intracellular ^{45}Ca content of longitudinal muscle strips from the guinea-pig small intestine. Tissues were pre-labelled with ^{45}Ca in standard medium (2·7 mM-K^+; *A*) or K^+-rich medium (108 mM-K^+; *B*) for 60 min and then placed for another 8 min in identical media (Co) or media containing SP, TEA, or high $[K^+]$. Means ± S.E. of means; $n = 7$–12; $*P < 0·05$ (two sample *t* test).

Intracellular ^{45}Ca content. While changes in the Ca influx rate can be followed by the 2 min pulse labelling method, it is also of importance to know whether or not an increase in the Ca influx rate results in an increased intracellular Ca content. As shown in Fig. 9*A*, exposure of strips to 22 nM-substance P, 30 mM-tetraethylammonium or 108 mM-K^+ for 8 min significantly increased the intracellular ^{45}Ca content, the increases being 1·13-fold for substance P, 1·21-fold for tetraethylammonium and 1·36-fold for high $[K^+]$.

Incubation of strips in 108 mM-K^+ solution as loading and test medium resulted in a 1·87 times higher intracellular ^{45}Ca content than in standard medium (Fig. 9*B*). Neither the presence of substance P (22 nM) nor that of tetraethylammonium (30 mM) for 8 min changed the intracellular ^{45}Ca content of strips depolarized with high $[K^+]$.

DISCUSSION

The results of this study indicate that there are two effects of substance P on the longitudinal muscle of the isolated small intestine of the guinea-pig, contraction and under certain conditions desensitization to substance P. Both effects were concentration-dependent and completely reversible. It appeared that desensitization only occurred when the time of exposure to substance P exceeded a certain time. The bell-shape of cumulative concentration–response curves for substance P was in all probability caused by desensitization which thus is a potential source of error in establishing concentration–peak response curves.

Fading and desensitization. The contractile response of the guinea-pig ileum to substance P was invariably followed by rapid fading of the contraction. The most plausible explanation of fading would be elimination of substance P from the organ bath but this explanation does not hold for the following two reasons. (i) After exposure of the ileum to 74 nM-substance P in the absence of gelatin (Fig. 5) the concentration of substance P in the bath decreased by 62% within 5 min. While according to the non-cumulative concentration–response relation for substance P (Fig. 1*A*) this should have reduced the contraction by only about 3%, the contraction was in fact reduced by 75% (Fig. 5). Hence it is evident that elimination of substance P could not have been the main cause of fading. Theoretically, elimination may be a more important factor for the fading of responses to substance P concentrations corresponding to the steep part of the concentration–response curve. However, it has been found that fading is negligible in isotonic responses to concentrations lower than the EC_{50} and that velocity and extent of fading increase with increasing concentrations of substance P (Holzer & Lembeck, 1980; see also Fig. 1*B*). Disappearance of substance P from the bath which occurred independent of the presence of tissue was most likely to be due to adsorption of the peptide to the bath wall. Adsorption of substance P to surfaces is a serious problem in handling this peptide as already noted by Mroz & Leeman (1979), Franco *et al.* (1979), and Huidobro-Toro *et al.* (1982), and the addition of gelatin to the bath medium as suggested by Mroz & Leeman (1979) or silanizing the bath proved ineffective in preventing this process. The time course of the contractile response to substance P might also be influenced to some degree by processes that would not become apparent in the radioimmunoassay, e.g. appearance of other biologically active substances in the organ bath or slow oxidation of substance P to substance P sulphoxide (Floor & Leeman, 1980). Appearance of substance P sulphoxide, which is much less active in contracting the ileum than substance P (Floor & Leeman, 1980), would have gone undetected by radio-immunoassay, since the antiserum used recognizes substance P and substance P sulphoxide to the same extent (Holzer, Bucsics, Saria & Lembeck, 1982). (ii) A second addition of substance P to the bath, after the response to the first exposure had largely faded away, failed to restore the initial peak contraction (Fig. 6) indicating that the ileum had in fact become less sensitive to substance P. As it is unlikely that substance P releases a physiological antagonist (Franco *et al.* 1979; Huidobro-Toro *et al.* 1982) it has to be inferred that fading reflects ongoing desensitization.

Basic assumptions on the mode of action of substance P. The discussion of the mechanisms involved in the contractile response and desensitization to substance P

has to be based on a number of premises. One of them is that the initial step by which substance P elicits contraction of intestinal muscle is binding to specific receptors which are constituents of the muscle cell membrane (Hanley & Iversen, 1980). Activation of the receptors by substance P depolarizes the muscle cell membrane, evokes slow waves, reduces the threshold depolarization required for generation of action potentials, and initiates or increases the frequency of action potentials (Fujisawa & Ito, 1982). There is circumstantial evidence that these effects are brought about by a reduction of the K^+ permeability of the cell membrane, but the permeability for other ions, particularly Ca^{2+}, Cl^- and Na^+, may also be changed (Bury & Mashford, 1976; Fujisawa & Ito, 1982; Holzer, 1982). The conductance changes induced by substance P in intestinal smooth muscle seem to be similar to those induced in neurones (Katayama *et al.* 1979; Konishi, Tsunoo & Otsuka, 1979; Dun & Minota, 1981; Hösli, Hösli, Zehntner & Landolt, 1981; Minota, Dun & Karczmar, 1981; Nowak & MacDonald, 1981).

Unlike the effect of substance P, the contractile effects of KCl and tetraethylammonium are most probably not mediated through specific membrane receptors. The action of KCl appears to be due to reduction of the potassium gradient across the cell membrane, the membrane potential being largely determined by the E_K (see Bolton, 1979). The major action of tetraethylammonium seems to be reduction of a potential-dependent K^+ conductance (Ito, Kuriyama & Sakamoto, 1970; Bolton, 1979; Tomita, 1981). While in this study tetraethylammonium was used to decrease the K^+ permeability of the muscle cell membrane, noradrenaline was employed to increase the K^+ permeability, which seems to be the main action of noradrenaline on intestinal smooth muscle (Ohashi, 1971; Bülbring, Ohashi & Tomita, 1981). The following discussion is also founded on the assumption that KCl, tetraethylammonium, and noradrenaline do not interfere with the kinetics of substance P binding to membrane receptors.

Desensitization of intestinal smooth muscle to substance P seems to be quite specific, since the contractile effects of acetylcholine, carbachol, dimethylphenylpiperazinium, histamine, 5-hydroxytryptamine, bradykinin or angiotensin II are not affected (Gaddum, 1953; Lembeck & Fischer, 1967; Franco *et al.* 1979; Monier & Kitabgi, 1980; Hutchinson & Dockray, 1981; Huidobro-Toro *et al.* 1982), whereas responses to KCl and tetraethylammonium are diminished as demonstrated in the present study. Most of the current explanations of specific desensitization assume that it is the consequence of receptor inactivation either due to the binding kinetics (Paton, 1961) or to conformational changes of the receptor molecule (Katz & Thesleff, 1957; Rang & Ritter, 1970), but the possibility has also to be considered that desensitization occurs in the chain of events which link receptor activation to contraction. In view of the assumptions made above, cross-desensitization between substance P, tetraethylammonium, and KCl might be due to blockade of a pathway in excitation–contraction coupling, which is activated by all three substances.

Possible mechanisms of action of substance P. The finding that tetraethylammonium increased while noradrenaline decreased the potency of substance P in contracting the longitudinal muscle are compatible with the contention that substance P acts, at least in part, by decreasing the K^+ permeability of intestinal smooth muscle. The situation is, however, complicated by the finding of unidirectional cross-desen-

sitization/sensitization between substance P and tetraethylammonium. Monitoring the efflux of ^{86}Rb (as a measure of membrane K^+ permeability; see Burgen & Spero, 1968; Bolton, 1981) showed that tetraethylammonium and substance P, in concentrations up to 100 times higher than those producing 50 % maximal contraction, slightly increased the ^{86}Rb efflux into the standard medium, but since tetraethylammonium as well as substance P depolarize the smooth muscle (Bolton, 1979; Fujisawa & Ito, 1982) it is not possible to draw any conclusion as to the effect on resting K^+ permeability. To the contrary, in K^+ depolarized strips both substance P and tetraethylammonium appreciably reduced the ^{86}Rb efflux indicating a reduction of a potential-sensitive K^+ conductance. With regard to tetraethylammonium, our results on intestinal smooth muscle are similar to findings obtained from vascular smooth muscle, in which tetraethylammonium greatly reduced the efflux of ^{42}K (Cooper, Goodford, Hardy, Herring, Hind & Keatinge, 1975; Casteels, Kitamura, Kuriyama & Suzuki, 1977).

In interpreting ion flux measurements a number of limitations of this approach should be borne in mind (see also Bolton, 1979; Brading, 1981). In particular, changes in the ion fluxes do not solely reflect changes in the membrane permeability for the respective ion, but can also result from effects on specific ion pumps which control the ionic distribution across the cell membrane. A difficulty exists also in relating substance P-induced changes in ion fluxes to the contractile effect, since there might be more than one type of receptor which mediate the myotropic effect of substance P (Hawcock, Hayes & Tyers, 1982; Lee, Iversen, Hanley & Sandberg, 1982). Consequently, changes in ion fluxes and contractile effect could be mediated by different substance P receptors. Another difficulty arises from the fact that the muscle strips used in this study contained the myenteric plexus, neurones of which are excited by substance P (Katayama *et al.* 1979). Tetrodotoxin was therefore used in all ion flux experiments to block propagated effects of substance P on neurones, but local effects may well have persisted. As to the direct effect of substance P on the smooth muscle, the presence of tetrodotoxin in only the ion flux experiments should not pose any problems for interpretation, since neither the membrane effects nor the contractile response to substance P in the smooth muscle are affected by tetrodotoxin (Fujisawa & Ito, 1982).

The effect of substance P in decreasing the ^{86}Rb efflux from depolarized muscle was clearly smaller than the effect of tetraethylammonium, the concentrations used being equiactive with regard to contraction. This strongly suggests that the contractile effect of substance P also involves a mechanism other than reduction of K^+ permeability. Evidence for this is further provided by the fact that although the decrease in ^{86}Rb efflux was sustained the contraction of substance P faded very rapidly, while the contractile response to tetraethylammonium was maintained as was the decrease in ^{86}Rb efflux. The findings of sustained changes in ^{86}Rb and ^{45}Ca fluxes may indicate that fading of the contractile response and desensitization to substance P do not result from receptor inactivation but occur at the level of excitation–contraction coupling. It would also appear that changes in the K^+ conductance are unlikely to account for desensitization.

Lowering the Ca^{2+} concentration reduced the ability of substance P to cause contraction of the ileum. This is in line with the findings of Bury & Mashford (1976)

that verapamil, which prevents Ca^{2+} from entering smooth muscle cells (Fleckenstein, 1977), or removal of extracellular Ca^{2+} suppresses the contractile effect of substance P. A possible interpretation of these results is that the contractile effect of substance P requires at least some influx of extracellular Ca^{2+}, a process which is sensitive to the action of verapamil (Bury & Mashford, 1976). In line with this is the observation of Fujisawa & Ito (1982) that the ability of substance P to enhance spike activity is lost in low medium $[Ca^{2+}]$.

Exposure of longitudinal muscle strips to 22 nM-substance P for 8 min first decreased and then increased the influx of ^{45}Ca as measured by 2 min pulse labelling. The initial decrease in ^{45}Ca influx is at present not understood, but the delayed increase in ^{45}Ca influx may be seen in context with the increased spike activity (Fujisawa & Ito, 1982), i.e. with the opening of potential-sensitive Ca^{2+} channels. Since the delayed increase in ^{45}Ca influx was also observed in K^+-depolarized strips it would seem that substance P can open Ca^{2+} channels which are not inactivated by depolarization.

The instant and sustained increases in ^{45}Ca influx brought about by tetraethylammonium and high $[K^+]$ (Fig. 8*A*) can simply be considered as a consequence of their action on K^+ conductance and E_K, respectively. Substance P, tetraethylammonium, and high $[K^+]$ all significantly enhanced the intracellular Ca^{2+} content during an 8 min exposure although the changes were small. Strips equilibrated in high $[K^+]$ showed a higher Ca^{2+} permeability and a higher intracellular Ca^{2+} content than in standard medium (see Mayer, van Breemen & Casteels, 1972). Although both substance P and tetraethylammonium produced a delayed increase in ^{45}Ca influx under this condition, the intracellular ^{45}Ca content remained unchanged indicating that extrusion of Ca^{2+} was also increased (see van Breemen, Wuytack & Casteels, 1975).

The finding that the contractile effect of substance P is decreased and the fading of the contraction increased in low extracellular $[Ca^{2+}]$ is also compatible with the idea that the effect of substance P involves the release of Ca^{2+} from a store which is in rapid equilibrium with the extracellular Ca^{2+}. A similar mechanism has been envisaged for the effects of carbachol and catecholamines on intestinal smooth muscle (see Fastier, Purves & Taylor, 1973; Ohashi, Takewaki, Shibata & Okada, 1975; Bülbring & Tomita, 1977; Bolton, 1979; Casteels & Raeymaekers, 1979; Brading & Sneddon, 1980). In this model, fading of the contractile response and desensitization to substance P would be accounted for by depletion of the Ca^{2+} store. To explain the relative specificity of the desensitization to substance P one would have to assume a Ca^{2+} store which is exclusively operated by the substance P receptors.

Is there any line of evidence which selectively points to such a mechanism of action of substance P? (i) The observations that desensitization developed only after a certain time and that extent and rate of recovery from desensitization depended on substance P concentration and contact time are equally accounted for by receptor inactivation or depletion of a Ca^{2+} store. (ii) The substance P-induced decrease of ^{86}Rb efflux from K^+-depolarized muscle strips remained unaltered when the medium $[Ca^{2+}]$ was lowered or raised (Table 1). Hence, provided that the same receptors mediate the decrease in ^{86}Rb efflux and contraction, substance P – receptor interaction appears to be rather Ca^{2+}-independent. If so, the findings that lowering the

extracellular Ca^{2+} concentration reduced the potency of substance P in contracting the ileum, accelerated fade and reduced the ability of the ileum to respond to a second addition of substance P after the response to the first addition had waned (see Fig. 6) are best explained with the Ca^{2+} store model. The findings would indicate that in low medium [Ca^{2+}] the Ca^{2+} store is smaller, more quickly emptied, and less effectively refilled than in standard medium, since Ca^{2+} influx may become rate-limiting. (iii) The existence of a Ca^{2+} store which is released by substance P could also offer an explanation for the observation that the ^{45}Ca influx was significantly decreased during the first 2 min of exposure to substance P. If one assumes that the Ca^{2+} store is in exchange with the intracellular Ca^{2+}, then a release of Ca^{2+} from the store would increase cytoplasmic [Ca^{2+}], reduce the inward gradient for Ca^{2+}, and consequently diminish Ca^{2+} influx.

The observation that fading of the contractile response to substance P was increased by noradrenaline, but decreased by tetraethylammonium, is difficult to explain, but may indicate an interaction between the effect of substance P on membrane K^+ permeability and the Ca^{2+}-dependent component of the action of substance P.

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